P2X7 receptors mediate resistance to toxin-induced cell lysis

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ABSTRACT

In the majority of cells, the integrity of the plasmalemma is recurrently compromised by mechanical or chemical stress. Serum complement or bacterial pore-forming toxins can perforate the plasma membrane provoking uncontrolled Ca^{2+} influx, loss of cytoplasmic constituents and cell lysis. Plasmalemmal blebbing has previously been shown to protect cells against bacterial pore-forming toxins. The activation of the P2X7 receptor (P2X7R), an ATP-gated trimeric membrane cation channel, triggers Ca^{2+} influx and induces blebbing. We have investigated the role of the P2X7R as a regulator of plasmalemmal protection after toxin-induced membrane perforation caused by bacterial streptolysin O (SLO).

Our results show that the expression and activation of the P2X7R furnishes cells with an increased chance of surviving attacks by SLO. This protective effect can not only be demonstrated in human embryonic kidney 293 (HEK) cells transfected with the P2X7R, but also in human mast cells (HMC-1), which express the receptor endogenously. In addition, this effect is abolished by treatment with blebbistatin or A-438079, a selective P2X7R antagonist. Thus blebbing, which is elicited by the ATP-mediated, paracrine activation of the P2X7R, is part of a cellular non-immune defense mechanism. It pre-empts

plasmalemmal damage and promotes cellular survival. This mechanism is of considerable importance for cells of the immune system which carry the P2X7R and which are specifically exposed to toxin attacks.

Abbreviations: HEK, human embryonic kidney 293 cells; HMC-1, human mast cell line 1; P2X7R, P2X7 receptor; RT, room temperature; SLO, streptolysin O

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1. INTRODUCTION

Serum complement or bacterial pore-forming toxins perforate the plasma membrane of a host cell and cause influx of extracellular - and efflux of cytoplasmic components, which might lead to cell death [1-4]. It is thus not surprising that eukaryotic cells have developed membrane repair mechanisms and adapted them to suit the nature and severity of plasmalemmal injury [5-8]. A breakdown of the Ca^{2+} concentration gradient between cytoplasm and extracellular milieu serves as an indicator of plasmalemmal disruption and acts as molecular guidance for the intracellular repair machinery. Intracellular [Ca^{2+}] elevations are recognized by Ca^{2+} sensors such as proteins of the annexin family, which bind Ca^{2+}-dependently to negatively-charged phospholipids [9] and have been ascribed a role in plasmalemmal repair [10-13].

Blebbing - a temporary detachment of the lipid bilayer from the submembranous cytoskeleton - is considered to be an early sign of intracellular Ca^{2+} elevation, which indicates imminent or incipient membrane injury [14]. We have recently proposed blebbing to be a damage-control mechanism, which is triggered after the failure of plasmalemmal resealing [15]. The ability to bleb provides a clear benefit of survival for a cell confronted with attacks on its plasmalemma by pore-forming toxins [15].

Since P2X7R activation is associated with an increase in membrane blebbing [16], we have investigated the role of this receptor as a molecular regulator of plasma membrane

protection after plasmalemmal perforation caused by the bacterial toxin SLO. The ionotropic P2X7R has two transmembrane domains; intracellular NH_2- and COOH-termini and forms a homotrimer [17]. It is ubiquitously expressed, with high levels in immune cells and exists as a multiprotein complex, including the non-muscle myosin heavy chains and other cytoskeletal elements [18, 19]. The activation of the P2X7R by extracellular ATP opens a cationic channel which gradually dilates to a larger pore [20-22]. It is itself subject to regulation by numerous polymorphic variants and isoforms which increase or decrease its efficiency [23]. In macrophages primed by lipopolysaccharide, channel opening is additionally associated with the secretion of pro-inflammatory cytokines IL-1β [24, 25] and IL-18 [26, 27]. P2X7R activation is followed by downstream effects such as blebbing [28, 29], microvesicle shedding [30, 31], cell fusion [32], proliferation [33] and eventually cell death [34]. Furthermore, in the absence of ATP, P2X7R plays a role in recognition and phagocytosis of foreign particles [35, 36].

We demonstrate that the expression of the P2X7R confers resistance to the attack by bacterial pore-forming toxins. This protective effect can be increased by activation of the P2X7R with ATP and is abolished by pretreatment with blebbistatin or A-438079, a selective P2X7R antagonist. We further suggest that the paracrine activation of this receptor by ATP, released by damaged neighbouring cells, initiates blebbing and thus induces resistance to toxin-induced cell lysis. Blebbing is a characteristic feature of injured cells. It is thus conceivable that cells – in particular within inflammatory foci – use this ATP-triggered, P2X7R-mediated warning system to alert their neighbours to an approaching wave of bacterial toxins.

2. MATERIALS AND METHODS

2.1. Cell culture

HEK cells were maintained in DMEM (Dulbecco's modified Eagle's medium), containing 2 mM glutamax, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (FCS) and were transfected as described [11, 37]. HEK cells stably transfected with the rat P2X7R (HEKrtP2X7) or the human P2X7R (HEKhuP2X7) [38] were cultured in DMEM/F12 medium supplemented with 2 mM glutamax, G418 (300 µg/ml), 2 mM MgCl2 and 10% FCS. HMC-1 were cultured in IMDM with 10% FCS, 100 U/ml penicillin and 100 µg/ml

streptomycin as described [39]. All cells were grown in 5% CO_2 at 37°C in a humidified incubator. For the viability experiments, the cells were challenged with 750 U/ml SLO from *Streptococcus pyogenes* (Sigma-Aldrich, Buchs, Switzerland) in Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES; pH = 7.4) containing 2.5 mM $CaCl_2$ or 1 mM EGTA (for the experiment without extracellular calcium) for 15 min at room temperature (RT). Cell viability was quantified by the cell health indicator alamarBlue® following the manufacturer instructions (Life Technologies, Carlsbad, USA [40]). To inhibit P2X7R-specific ATP stimulation, the cells were incubated with 1 μM or 10 μM A-438079 (Sigma-Aldrich, Buchs, Switzerland) for 15 min at RT.

2.2. Expression of annexins as fusions with fluorescent proteins

The coding sequences of annexin A1 and A2 were cloned into the Living Colors Fluorescent protein vectors pEGFP-N1 or pEYFP-N1 (Clontech, Saint-Germain-en-Laye, France) following PCR amplification from human bladder smooth muscle cDNA. The fluorescent protein was attached to the C-terminus of the annexins, which has been shown not to affect the protein function [41]. The oligonucleotide pairs used for PCR correspond to the N-termini (forward) and C-termini (reverse, lacking the stop codon) of annexins and contain appropriate restriction sites to allow the in-frame cloning with fluorescent proteins.
For annexin A1, 5'-TTTAAGCTAGCG**ATG**GCAATGGTATCAGATTCCTC-3' forward and 3'TTATTCTGCAGGTTTCCTCCAAACAGAGCCAC-5' reverse.
For annexin A2, 5'-AATATAGCTAGC**ATG**TCTACTGTTCACGAAATCCTG-3' forward and 5'ATAAACTCGAGTTAGTATAGGCTTTGACAGAC-3' reverse.

2.3. Imaging

Glass coverslips with annexin-expressing HEK cells were mounted in a perfusion chamber at RT in Tyrode's buffer containing 2.5 mM $CaCl_2$. To induce blebbing, the ATP analogue 2'(3')- O-(4-Benzoylbenzoyl)ATP (BzATP) was added to the cells at concentrations between 10 μM and 100 μM (in Tyrode's buffer). Cell lysis was monitored by irreversible elevation of intracellular [Ca^{2+}] above 20 μM using permanent plasmalemmal translocation of annexin A1 as a read-out [15]. Cells were challenged with 750 U/ml SLO in Tyrode's buffer for 15 min at RT. When indicated, the cells were pretreated with blebbistatin (100

μM, 30 min) and/or ATP (30 μM, 10-15 min) before the addition of SLO. The fluorescence was recorded in an Axiovert 200M microscope with a laser scanning module LSM 510 META (Carl Zeiss AG, Feldbach, Switzerland) using a 63x oil immersion lens. The images were analyzed using the 'Zeiss LSM Image examiner' software package (Carl Zeiss AG). To determine the percentage of lysis, the number of cells with annexin A1 translocation was divided by the total cell number [15]. Transient plasmalemmal translocations of fluorescently-labeled annexin A2 were used to monitor elevations of intracellular Ca^{2+} in the low micromolar range [11, 42].

Numerical data are expressed as mean values together with the standard error. The statistical analyses were performed using GraphPad Prism 5.04 and Microsoft Excel 2010. The level of significance was set at $p<0.05$. Significant differences are marked with asterisks.

2.4. Western blotting

HEK, HEKrtP2X7 and HEKhuP2X7 cells were resuspended in SDS sample buffer (200 mM Tris–HCl, 40 mM EGTA, pH 7, 3.1% SDS, 7.7% β-mercaptoethanol, 13.4% glycerol and 60 μg/ml of bromophenol blue) and boiled for 1 min. Samples were run on 8% SDS-polyacrylamide minigels together with a molecular weight marker (Page Ruler Prestained, Thermo Scientific, Erembodegem, Belgium). Equal amounts of protein were loaded for the different protein extracts as estimated by a test gel. Blotting was carried out overnight onto a PVDF membrane (Millipore AG, Zug, Switzerland). Unspecific binding sites were blocked with 4% non-fat dry milk in low salt buffer (0.9% NaCl, 10 mM Tris-Base, 0.1% Tween 20) for 1 h at RT. Primary antibodies (polyclonal rabbit-anti-P2X7, Alomone Labs, Jerusalem, Israel) were diluted in low salt buffer supplemented with 2% non-fat milk powder and incubated for 1 h at RT. The blot was washed 3 times with low salt buffer for 10 min. Secondary antibodies (HRP-donkey-anti-rabbit IgG, GE Healthcare, Glattbrugg, Switzerland) were diluted in low salt buffer supplemented with 2% non-fat milk powder and incubated for 45 min at RT. Finally, the blot was washed again 3 times with low salt buffer for 10 min. Chemiluminescence reaction was performed using Western Bright ECL from Advansta (Menlo Park, CA, USA) and the signal was detected by a Fusion FX imaging system (Vilber Lourmat, Marne-la-Vallée, France).

3. RESULTS

In order to investigate P2X7R function in plasmalemmal repair, we initially applied a well characterized model system (HEK cells), in which blebbing has previously been associated with increased survival [15]. We confirmed that the transcription (Figure 1A) and protein expression (Figure 1B) of P2X7R occurred in HEK cells stably transfected with the P2X7R gene, but not in control cells. Immunostaining with antibodies against the P2X7R demonstrated that it was localized to the plasmalemma, as previously described, whereas untransfected HEK cells were devoid of signal (Figure 1C).

The activation of a functional P2X7R leads to an increase in [Ca^{2+}], followed by blebbing [43, 44]. Therefore, the functionality of the P2X7R in stably transfected HEK cells was established by stimulation with increasing concentrations of BzATP and by simultaneous monitoring of blebbing activity and [Ca^{2+}] by Ca^{2+}-dependent plasmalemmal translocation of fluorescently labeled annexin A2 [11].

HEKrtP2X7 cells responded to increasing concentrations of BzATP by an increased plasmalemmal blebbing (Figure 2A and Supplementary Movie 1). After stimulation with 10 μM BzATP, 2.4±1.8% of the cells blebbed, whereas at 30 μM BzATP, ~ 50% of the cells displayed blebbing during 1 h of treatment. At a concentration of 100 μM BzATP, almost all cells blebbed (98.6±1.4%; 450 cells on 30 slides).

Annexin A2 reversibly translocates between the cytoplasm and the plasmalemma at low micromolar [$Ca^{2+}]_i$ [42]. In non-stimulated cells, annexin A2 localizes within the cytoplasm [11]; this pattern was not altered by the expression of the P2X7R. Blebbing closely correlated with annexin A2 membrane translocation, confirming Ca^{2+} influx through the opened P2X7 channel after stimulation with BzATP (Figure 2B). Stimulation with 30 μM BzATP led to increased blebbing (Figure 2C) and plasmalemmal translocation of annexin A2 (Figure 2D) in 64.5±8.1% of cells (495 cells on 33 slides).

In order to determine the role of the P2X7R in cellular survival, toxin-induced lysis was analyzed by monitoring irreversible membrane translocation of YFP-annexin A1 ([$Ca^{2+}]_i$ – elevation above 20 μM) [15]. Figure 3A shows that the expression of the P2X7R alone confers resistance against SLO toxicity. HEKrtP2X7 cells showed a significantly lower amount of SLO-induced lysis (59.5%±6.4%, *t*-test: P<0.001; 147 cells on 11 slides) compared to the wild-type cells (100%±4.8%, 187 cells on 13 slides). As expected, the P2X7R stimulation with ATP prior to toxin incubation led to an additional reduction of lysis (Figure 3B; 24.4%±7.7%, *t*-test: P<0.01, 277 cells on 14 slides). Representative confocal

micrographs depict the extent of annexin A1 translocation during the experimental conditions described above (Figure 3C).

To elucidate the role of Ca^{2+} in the protective effect of the P2X7R, we performed alamarBlue® cell viability assays [40]. In the presence of extracellular Ca^{2+}, an increased viability was observed in HEKrtP2X7 cells compared to non-transfected HEK cells (Figure 3D). Correspondingly, we could detect significantly more blebbing in HEKrtP2X7 cells compared to HEK cells (44.5±9.9% versus 27.1±4.7%) after SLO-treatment (Figure 3E). Such protective effect was lacking in the absence of extracellular Ca^{2+} and the viability after treatment with SLO at Ca^{2+}-free conditions was drastically reduced in both cell types. Our data demonstrate that the P2X7R has a protective effect, which is dependent on the extracellular [Ca^{2+}], even without activation by ATP. Ca^{2+} ions can enter the cells through the SLO pores and trigger blebbing by activating contraction of myosin II [46]. The increase in blebbing in HEKrtP2X7 cells compared to HEK cells even without stimulation by ATP may be a consequence of the increased ability of the endoplasmatic reticulum (ER) to accumulate, store and release Ca^{2+} [47]. Consequently, the Ca^{2+} influx via toxin pores might potentiate Ca^{2+} release from the ER, leading to a larger increase in cytosolic [Ca^{2+}] in P2X7 transfected cells resulting in stronger blebbing.

We next investigated whether membrane blebbing was responsible for the protection against lysis. Blebbing - and its concomitant positive effect on cellular survival mediated by the P2X7R - was inhibited by pretreatment with blebbistatin (Figure 4A**;** 513 cells on 27 slides; Figure 4B), an agent that is known to interact with non-muscle myosin II and to inhibit blebbing [48].

To determine if ATP is a P2X7R-specific mediator of this process, we treated HEKrtP2X7 cells with A-438079, a selective P2X7R antagonist, which reversibly blocks rat and human P2X7R and inhibits BzATP induced IL-1β release as well as pore formation [49]. Pretreatment with A-438079 before the incubation with ATP (30 μM) and SLO treatment, led to a complete abolishment of the ATP-mediated protective effect (Figure 5A; 33 measurements in 3 independent AlamarBlue® assays).

The physiological significance of our findings were emphasized in experiments with HMC-1, which express the P2X7R endogenously [50]. Viability was significantly increased after pretreatment of the mast cells with ATP prior to toxin exposure (Figure 5B; from 17.7±1.3% to 32.1±1.6%; 34 measurements in 3 independent assays) and abolished by pretreatment with A-438079.

4. DISCUSSION

The P2X7R has been proposed to function as a regulator of inflammation [51]. In systemic illnesses such as rheumatoid arthritis, the activation of the P2X7R is associated with an increase in the secretion of proinflammatory cytokines and an exacerbation of the disease [52]. Its prolonged activation leads to apoptotic cell death [53] and its absence has been linked to a diminished output of inflammatory mediators such as IL-1 [54]. ATP is considered a physiological ligand of the P2X7R, which *in vivo* is released from viable or dying cells or from degranulating platelets [55-57]. In view of its effect on neighbouring cells, ATP has been identified as an endogenous danger signal with potentially detrimental effects when it is released into the extracellular space after tissue damage [58].
Numerous studies have addressed the frequently deleterious consequences for an organism, which is flooded with proinflammatory cytokines following P2X7R activation [52, 58]. Conversely, the absence or loss-of-function of the P2X7R has been shown to diminish cellular host defenses against pathogens such as *Chlamydia* [59] or *Toxoplasma gondii* [60].
Our study addresses the role of P2X7R activation in the survival of cells subjected to an attack by bacterial toxins. Exposed to an often unfavorable environment and threatened by the exposure to bacterial toxins, cellular survival is critically dependent on an early warning system in combination with efficient plasmalemmal repair.
Plasmalemmal repair can be achieved by different Ca^{2+}-dependent mechanisms: eukaryotic cells are capable of patching injured regions with internal membranes transported to the surface of the cell by exocytosis [5] or of resealing the plasma membrane by endocytosis of the damaged sites [6, 61]. Microparticle shedding has been shown to play a role in the quarantining and elimination of injured plasmalemmal regions [8, 62].
Previous studies have associated enhanced P2X7R activity with plasmalemmal blebbing [21, 63] and microvesicle shedding [30] as well as with the death of injured cells [14, 64]. In addition, blebs play also a role in cellular migration [46, 65, 66]. We have recently proposed that blebs confer resistance to toxin-induced plasmalemmal injury by acting as a trap for the damaged membrane region, which is sealed off from the cell body [15].
Proteins of the annexin family are able to sense elevated $[Ca^{2+}]_i$ [42]. In the presence of Ca^{2+}, annexin A1 aggregates and fuses biological membranes [9, 67-69]. Exposed to a strong flux of Ca^{2+} in the vicinity of a toxin pore, annexin A1 binds to the plasmalemma within the bleb and cross-links the adjacent membranes of its slender neck, thus forming a

plug that creates two separate compartments. This process enables the cell to repair its plasmalemma within the bleb without endangering the cell body. After membrane integrity has been restored and Ca^{2+} homeostasis has been re-established, annexin A1 translocates back to the cytoplasm thereby dissolving the plug [13].
Stimulation of the P2X7R (by ATP or its analogue BzATP) leads to the opening of a cation channel [21] and Ca^{2+} influx, which initiates blebbing [28] and microparticle release [30]**.** Our data show that the expression of the P2X7R alone improves the survival of cells during attacks by the pore-forming bacterial toxin SLO. This protective effect is even more pronounced if the receptor is stimulated with its physiological ligand ATP prior to toxin exposure. In addition, this effect can be inhibited by pretreatment with blebbistatin or a specific P2X7R antagonist.

5. CONCLUSIONS

Plasmalemmal blebbing constitutes an intricate cellular defense system: on the one hand, blebbing allows the cell to isolate the damaged plasmalemmal segment and protects the cell body from further injury by restricting the elevated intracellular [Ca^{2+}] to the blebs [15], on the other hand, the release of ATP from the injured cell acts as a paracrine stimulus and induces blebbing by activating the P2X7 receptors of cells in the vicinity (Figure 6). Our study underlines the important role of P2X7R-induced blebbing as part of a non-immune defense mechanism against bacterial pore-forming toxins. Since the P2X7R is particularly prevalent in cells of the immune system [70], this mechanism warns and shields those cells, which are in the first line of toxin attacks.

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FIGURE LEGENDS

Figure 1. Expression and localization of the P2X7R in stably transfected HEK cells. (A) qRT-PCR analysis of P2X7R expression confirms its presence only in the stably transfected cell line (rtP2X7). (B) Western blot analysis of HEK (1-2), HEKrtP2X7 (3-4) and HEKhuP2X7 (5-6) cells shows the P2X7R to be present only in the transfected cell lines (column 3-6). β-actin was used as loading control. (C) A confocal micrograph of HEKrtP2X7 cells (left picture) immunolabeled with an antibody against the P2X7R (green) and DAPI (red) reveals the plasmalemmal localization of the receptor. HEK cells (right picture) do not show any membrane labeling. Scale bar = 10 μm.

Figure 2. Blebbing and annexin A2 translocation are induced in HEKrtP2X7 cells treated with BzATP. (A) Stimulation of the P2X7R by BzATP induces blebbing in a concentration dependent manner (N = 30). (B) Plasmalemmal translocation of annexin A2 after BzATP treatment (N = 33). Asterisks = significant difference compared to the results from the 100 μM experiment in each graph. (C) Representative confocal micrographs show GFP-transfected HEKrtP2X7 cells before (upper picture) and after stimulation with 100 μM BzATP (lower picture), where blebbing is clearly visible. (D) The cytoplasmic localization of annexin A2-GFP (Anx2-GFP) in transfected HEKrtP2X7 cells before stimulation with 100 μM BzATP (upper picture) and its membrane translocation after stimulation with 100 μM BzATP (lower picture). Treatments were performed for 1 h at RT. Scale bar = 20 μm.

Figure 3. The expression of the P2X7R in HEK cells confers resistance against SLO treatment. Lysis was analyzed by the annexin A1 translocation assay. (A) HEKrtP2X7 cells treated with SLO for 15 min show a significantly lower amount of lysis compared to HEK cells (HEK: N = 22, HEKrtP2X7: N = 26 per experimental group; t-test: $P<0.001$). (B) ATP stimulation protects HEKrtP2X7 cells against lysis by SLO. Pretreatment of HEKrtP2X7 cells with 30 μM ATP for 10 min leads to a significant additional reduction of lysis. N = 6 per experimental group, t-test: $P<0.01$. (C) Confocal micrographs of HEK and HEKrtP2X7

cells transfected with YFP-labeled annexin A1 after SLO treatment for 15 min (HEK = HEK cells; P2X7 = HEKrtP2X7 cells; +ATP = 30 μM ATP pretreatment for 15 min). Scale bar = 20 μm. (D) AlamarBlue® viability assays of HEK and HEKrtP2X7 cells after SLO treatment for 15 min without extracellular calcium (no [Ca^{2+}]) and with 2.5 mM extracellular calcium (2.5 mM [Ca^{2+}]). (E) Percentage of blebbing during incubation with SLO for 15 min in HEK and HEKrtP2X7 cells. The transfected cells show significantly more blebbing. The extracellular calcium concentration was adjusted to 2.5 mM in (A)-(C) and (E). All treatments were performed at RT. Asterisks = significant differences compared to the left bars.

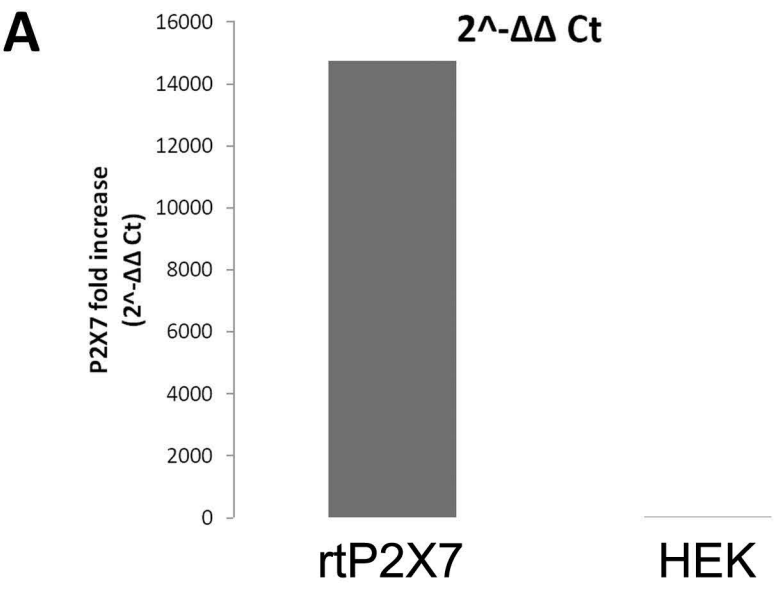
Figure 4. Blebbistatin treatment abolishes the protective effect of ATP stimulation during toxin attack by SLO. (A) In HEKrtP2X7 cells, pretreatment with blebbistatin (100 μM) for 30 min leads to a reversal of the protective effect in cell lysis caused by treatment with ATP (30 μM, 15 min). Ctrl: 45 min incubation with Tyrode's buffer; ATP: 30 min Tyrode's buffer + 15 min ATP (30 μM) in Tyrode's buffer; bleb: 30 min incubation with blebbistatin (100 μM) + 15 min ATP (30 μM), both in Tyrode's buffer with 2.5 mM [Ca^{2+}]. All treatments were followed by incubation with SLO for 15 min. N = 9 per experimental goup, t-test: $P<0.01$ (*ctrl - ATP*); $P<0.05$ (*ATP - bleb*). (B) Confocal micrographs of HEKrtP2X7 cells transfected with YFP-labeled annexin A1 after SLO treatment (ctrl = no additional pretreatment; ATP = 30 μM ATP pretreatment for 15 min; bleb: pretreatment with 100 μM blebbistatin for 30 min + 30 μM ATP for 15 min). All treatments were performed at RT. Scale bar = 20 μm.

Figure 5. Pretreatment with a P2X7R specific antagonist (A-438079) results in abolishment of the protective effect induced by ATP. (A) HEKrtP2X7 cells pretreated with A-438079 (1 μM) for 15 min before the incubation with ATP (30 μM for 15 min) and SLO show a complete abolishment of the protective effect induced by ATP. N = 33, $P<0.01$. (B) Stimulation of HMC-1 cells with ATP (20 μM for 25 min) leads to improved resistance against SLO treatment (17.7% to 32.1% viability) and is abolished by pretreatment with A-438079. All treatments were performed at RT. N = 43, t-test: $P<0.01$, asterisks denote significant differences. Data obtained by alamarBlue® viability assays.

Figure 6. The role of blebbing as a non-immune defense mechanism. Blebbing provides spatially confined compartments in which membrane repair can take place (left cell). ATP

released from damaged cells serves as a paracrine signal, alerting neighbouring cells by activation of the P2X7R followed by blebbing (right cell).

Figure 1


A
2^-ΔΔ Ct
P2X7 fold increase (2^-ΔΔ Ct)
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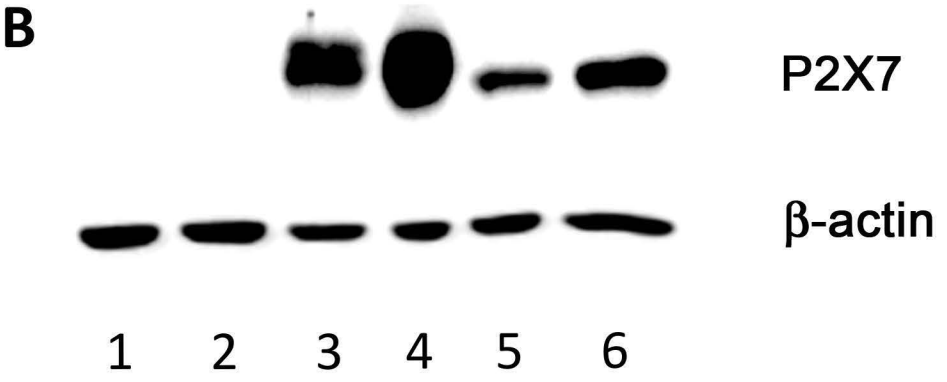
14000
12000
10000
8000
6000
4000
2000
0
rtP2X7
HEK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P2X7
β-actin
1
2
3
4
5
6

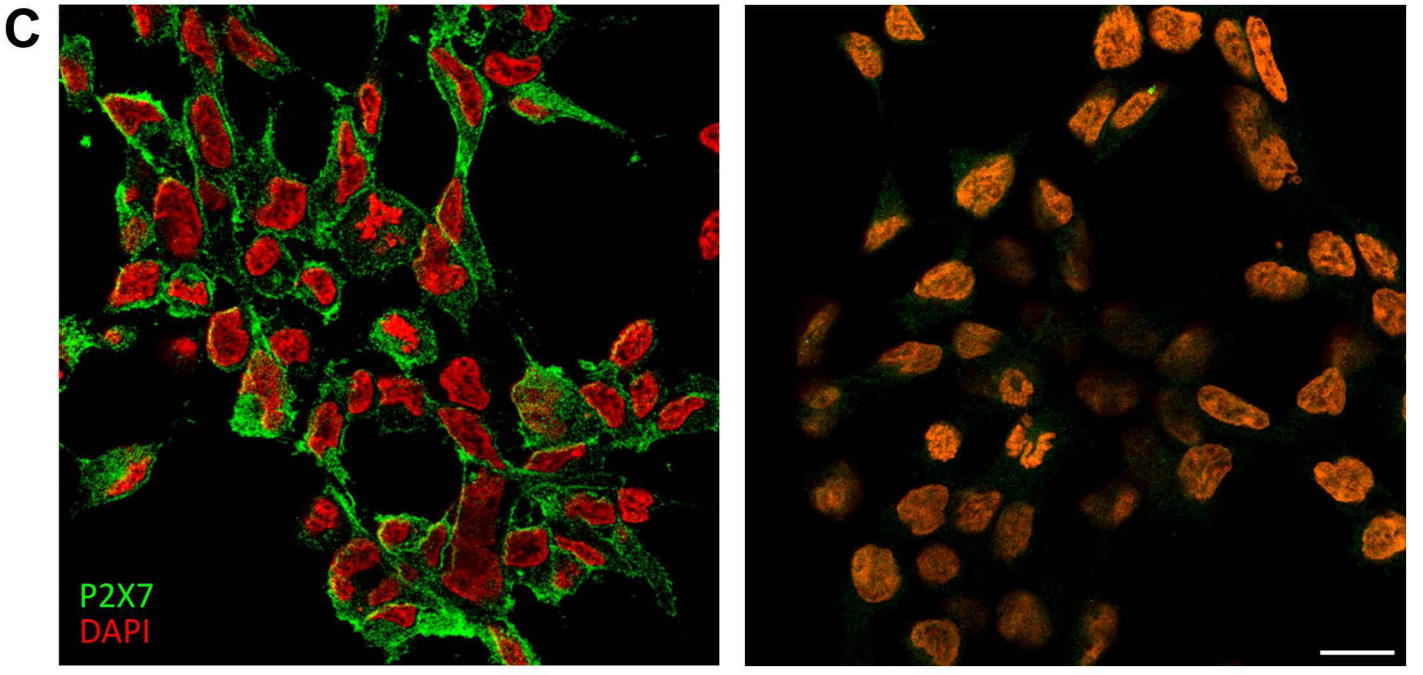
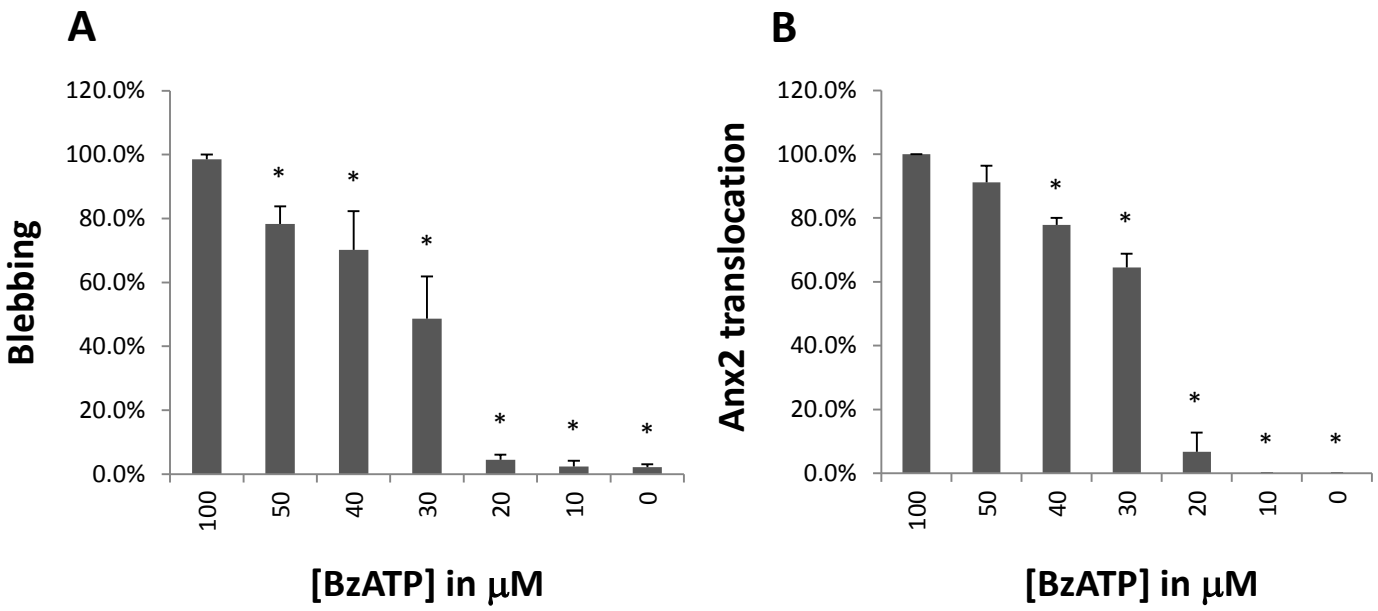
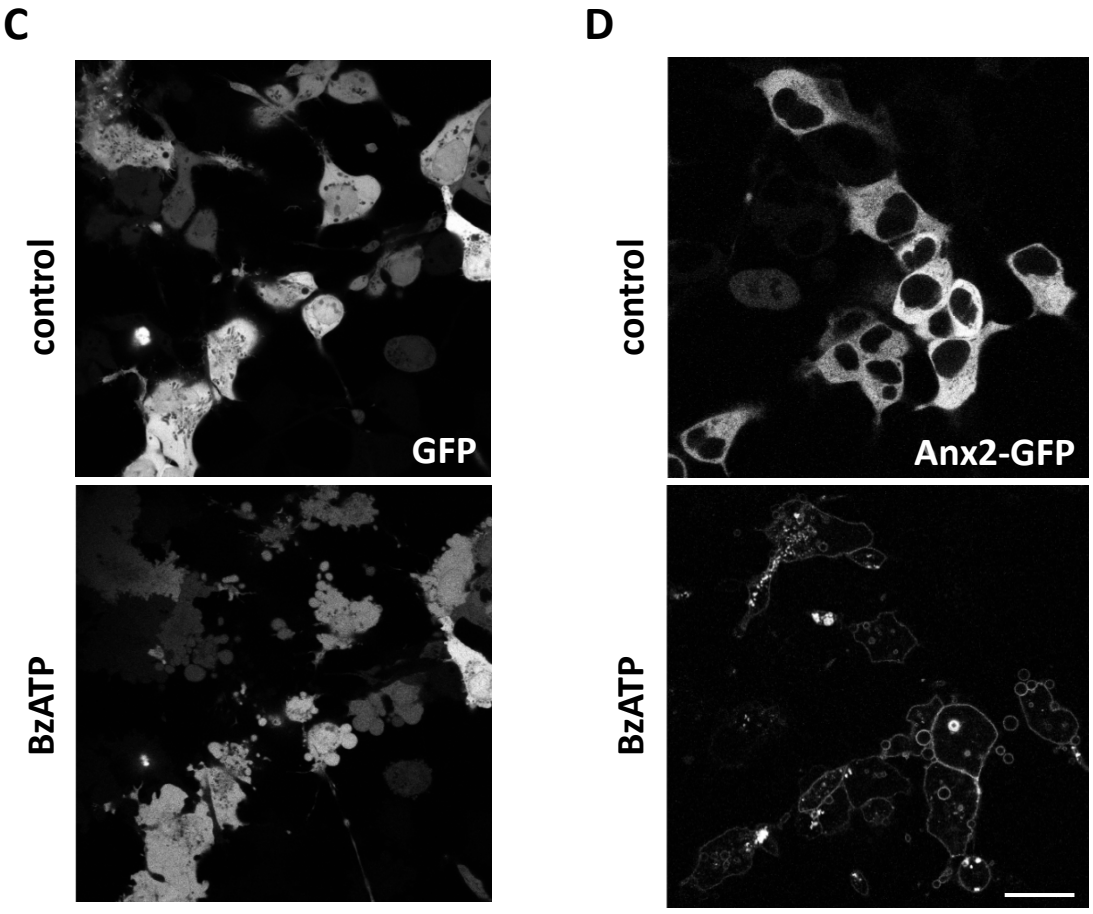

C
P2X7
DAPI

Figure 2


A
Blebbing
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
100
50
40
30
20
10
0
[BzATP] in μM
B
Anx2 translocation
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
100
50
40
30
20
10
0
[BzATP] in μM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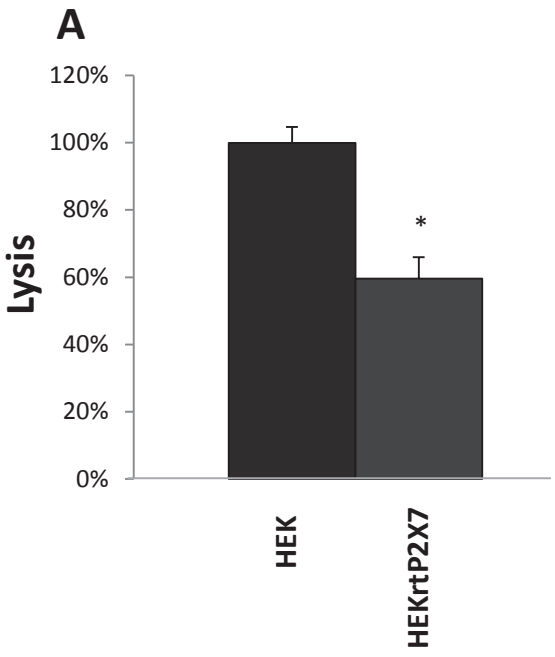
control
GFP
BzATP
D
control
Anx2-GFP
BzATP

Figure 3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120%
100%
80%
60%
40%
20%
0%
Lysis
*
HEK
HEKrtP2X7

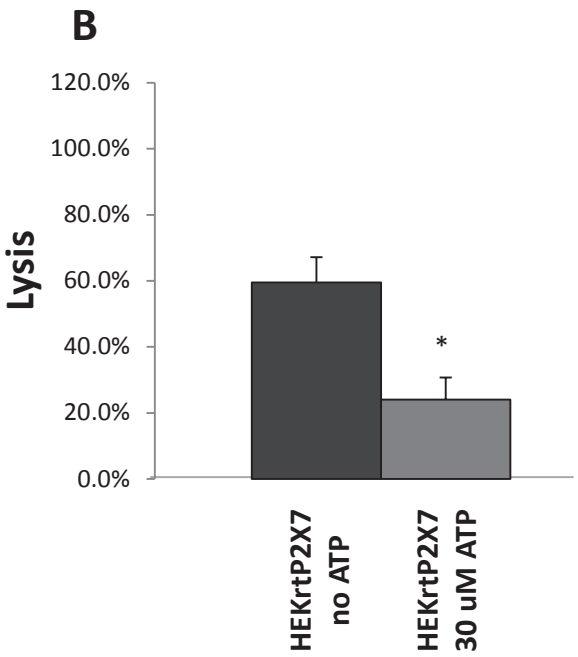
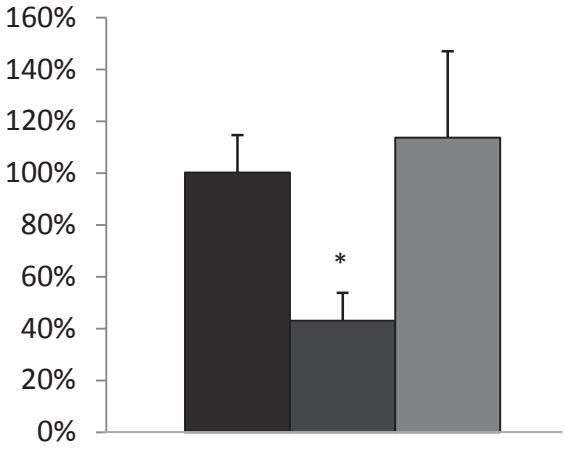

B
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
Lysis
*
HEKrtP2X7 no ATP
HEKrtP2X7 30 uM ATP

Figure 4

A



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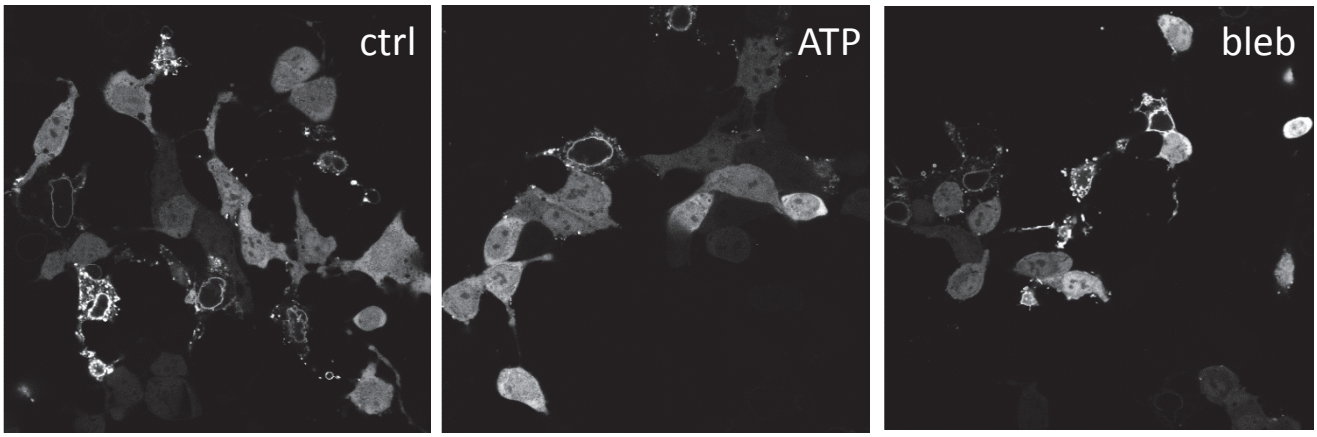
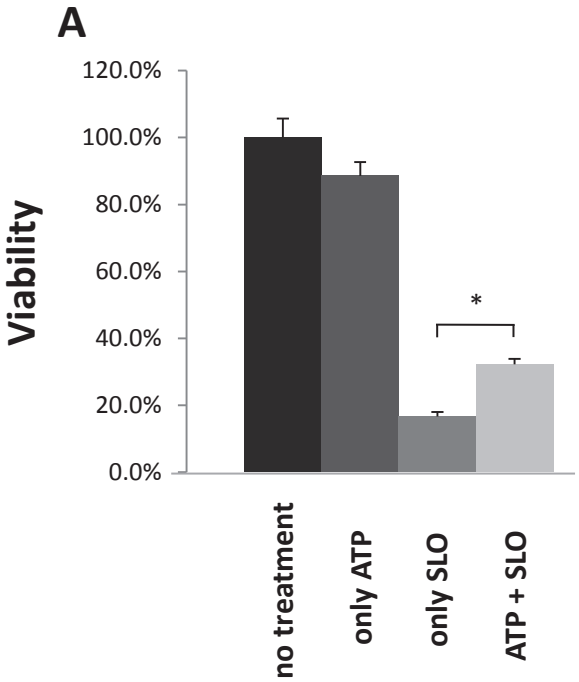


Figure 5


A
Viability
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
*
no treatment
only ATP
only SLO
ATP + SLO

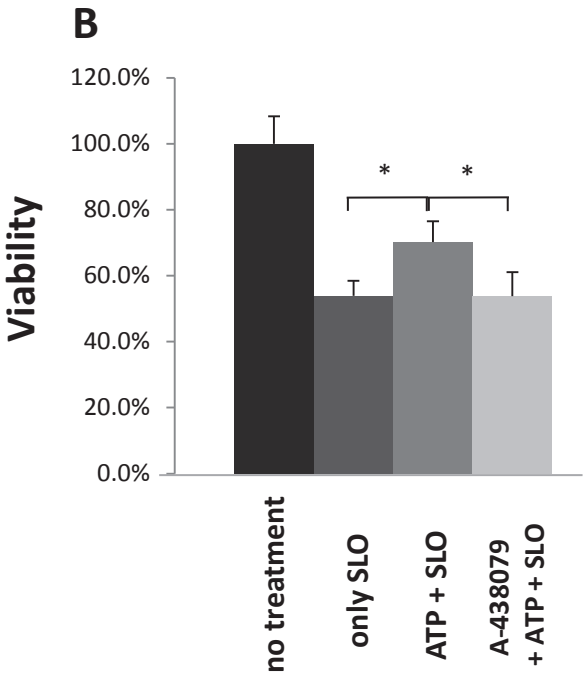

B
Viability
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
*
*
no treatment
only SLO
ATP + SLO
A-438079
+ ATP + SLO

Figure 6